



Activin signals via SMAD2/3 between germ and somatic cells in the human fetal ovary and regulates kit ligand expression

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Abstract

Ovarian germ cell survival is dependent upon the formation of primordial follicles, which occurs during fetal life in the human. Activin contributes to germ cell proliferation and survival at this time. SMADs2 and 3 are central elements in the activin signalling pathway and thus indicate sites of activin action. We have investigated the expression and localisation of SMADs2 and 3 in the fetal ovary between 14 and 20 weeks gestation, i.e. preceding and during primordial follicle formation. *SMAD3* mRNA expression increased 1.9 fold ($P=0.02$). SMAD2 and 3 proteins were localised by immunofluorescence to the nuclei of three distinct populations of somatic cells: (a) stromal cells between clusters of germ cells; (b) some somatic cells intermingled with activin β A-expressing germ cells; (c) pre-granulosa cells surrounding primordial follicles. Germ cells did not express SMAD2 or 3. Activin A increased and follistatin decreased phosphorylation of SMAD2/3 in vitro, and activin increased *SMAD2* and decreased *KITLG* mRNA expression. It therefore appears that somatic cells are the targets for activin signalling in the developing ovary. The effects of activin on germ cells are indirect and include mediation by the kit ligand/c-Kit pathway, rather than being an autocrine germ cell effect. © 2007 Elsevier Inc. All rights reserved.

Keywords: Fetal ovary; Smad2/3; Activin; Kit ligand; c-Kit; Primordial follicle

Introduction

The key step in germ cell survival during ovarian development is the formation of primordial follicles, these then determining the reproductive lifespan of the ovary. In human, they are formed during fetal life, from approximately 18 weeks of gestation. Following migration of primordial germ cells (PGCs) from the extraembryonic mesoderm of the yolk sac to the genital ridge, sexual differentiation occurs at approximately 8 weeks gestation (i.e. 6 weeks post conception) in the human. Initially the germ cells undergo mitotic proliferation, reaching maximum numbers at around mid gestation (Baker, 1963). During proliferation germ cells form syncytial nests with

intermingling somatic cells and are surrounded by a meshwork of ovarian stroma (Pepling and Spradling, 2001; Sawyer et al., 2002). Following entry into meiosis, germ cell loss through apoptosis (De Pol et al., 1997; Vaskivuo et al., 2001) results in less than 20% surviving to birth (Baker, 1963). Oocytes become individually surrounded by somatic cells forming primordial follicles (Hirshfield, 1991) which determines oocyte survival and thus protects against apoptosis (Fulton et al., 2005). Both cell types are believed to play an active role in the process of follicle development, but the molecular mechanisms regulating this interaction are still largely unknown. However, a number of key genes have been identified as being required for the formation of a normal complement of primordial follicles (Matzuk, 2000; Amleh and Dean, 2002; Skinner, 2005). These include growth factors and their receptors, e.g., kit ligand and its receptor c-Kit (Manova et al., 1993; Richards, 2001; Robinson et al., 2001; Klinger and De Felici, 2002), the neurotrophin receptor TrkB (Disen et al., 1995; Ojeda et al., 2000; Anderson

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et al., 2002; Spears et al., 2003) and oocyte-specific transcription factors (Dean, 2002; Bayne et al., 2004; Rajkovic et al., 2004).

Activin is one of several identified transforming growth factor β (TGF β) family members with major roles in folliculogenesis (Findlay et al., 2002; Knight and Glistler, 2003, 2006; Shimasaki et al., 2004). Members of this super-family can influence several aspects of cell lineage and activity including proliferation, differentiation, adhesion, motility and apoptosis. Many are involved in embryonic development as well as in adult tissue homeostasis (Mishina et al., 1999; Padgett and Patterson, 2001). Activin subunits, but not the inhibin α subunit, are present in the developing human fetal ovary, and activin may contribute to the regulation of germ cell survival and proliferation (Martins da Silva et al., 2004). The activin β A subunit is expressed by clusters of germ cells immediately preceding primordial follicle formation, but not by oocytes within primordial follicles (Martins da Silva et al., 2004). Activin β A expression therefore appears at a stage when oocytes are not expressing c-Kit after entry into meiosis, but is reduced when oocyte expression of c-Kit reappears at diplotene, i.e., at follicle assembly (Driancourt et al., 2000; Hutt et al., 2006). In the mouse, follicle assembly occurs in the neonatal period: treatment with activin during this time increased the number of primordial follicles that formed (Bristol-Gould et al., 2006). Activin signals through membrane-bound serine–threonine kinase receptors, which are also expressed in the developing ovary. Activin binds to a type II receptor which recruits and phosphorylates a type I receptor, ALK4, which in turn phosphorylates Smad2 and Smad3 proteins (Massague, 1998). Smad proteins are central elements in the activin receptor signalling pathway: following activation by phosphorylation they dimerise with Smad4 and translocate to the nucleus to modulate target gene expression. Smads2 and 3 can also mediate signalling by other TGF β family members and thus are not exclusive to activin (Massague, 1998). Smad2 and Smad3 have both been implicated as important factors in ovarian development and function (Chang et al., 2002; Drummond et al., 2003). It has also been demonstrated that *Smad3* deficient (*Smad3*^{−/−}) mice have reduced fertility (Tomic et al., 2002, 2004) and show deficient granulosa cell proliferation. Identification of cell types expressing SMADs2 and 3 in the nucleus therefore provides information as to the targets of activin or other TGF β factor signalling. We have made use of this in the present study to identify the cells in the developing human ovary which respond to and mediate the effects of activin on germ cell survival. Furthermore we identify kit ligand (*KITLG*), a key regulator of mammalian ovarian development (Driancourt et al., 2000; Hutt et al., 2006), as a target of activin-regulated transcriptional repression in the human fetal ovary.

Materials and methods

Tissue

Human fetal ovaries were obtained following medical termination of pregnancy. Consent was obtained in accordance with national guidelines (Polkinghorne, 1989) and the study was approved by the Lothian Research Ethics Committee. Pregnancies were terminated by treatment with mifepristone

(200 mg orally) followed 48 h later by misoprostal (800 mcg) three hourly per vaginam. Ultrasound examination was used to determine gestation prior to termination and this was subsequently confirmed by direct measurement of foot length. Pregnancies were all terminated for social reasons and all fetuses appeared morphologically normal.

Ovaries were removed from the fetus and processed in 3 ways. Ovaries were either snap frozen and stored at -70°C , fixed in Bouins solution for 2 h followed by transferring to 70% ethanol before processing into paraffin using standard methods, or prepared for tissue culture as described below.

Isolation of RNA and synthesis and amplification of cDNA

Total RNA was extracted from snap frozen samples of fetal ovary (14–20 weeks) or cell culture pellets/lysates (see below). This was performed using the RNeasy Mini Kit (Qiagen, Crawley, UK) with on-column DNase digestion according to the manufacturer's instructions. Reverse transcription was performed using Expand Reverse Transcriptase (Roche Diagnostics, Lewes, UK). Briefly, 1 μg total RNA was incubated with 50 pmol oligo (dT)₁₆ primer for 10 min at 65°C and then placed on ice. A reaction mix comprising buffer, 10 mmol/l Dithiothreitol, 1 mmol/l of each of deoxynucleotide triphosphate (dNTP), RNasin ribonuclease inhibitor (Promega, Southampton, UK) and 50 IU reverse transcriptase (RT⁺), was added to each tube in a total volume of 20 μl . Equivalent reactions without reverse transcriptase provided RT-controls. All tubes were then incubated at 42°C for 1 h.

Amplification of SMAD2 and 3 cDNA by PCR

Target-specific PCR was performed using 1 μl of the RT⁺ or RT[−] cDNA synthesis reactions or water as template in a reaction volume of 25 μl containing 2.5 IU of HotStar Taq DNA polymerase (Qiagen, Crawley, UK), 2.5 μl 10 \times reaction buffer giving a final concentration of MgCl₂ of 1.5 mmol/l, 200 $\mu\text{mol/l}$ dNTP, and 500 nmol/l of appropriate forward and reverse primers. PCR amplification conditions consisted of an hot start step at 95°C for 15 min to activate the enzyme, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s and extension at 72°C for 30s; a final extension period at 72°C for 10 min completed the amplification. *SMAD2* and 3 primers were designed to span an intron to ensure that genomic DNA was not amplified. Primers for the constitutively expressed gene *GAPD* were used to confirm the integrity of the RNA and efficacy of the PCR reaction. Primers (MWG, Ebersberg, Germany) were: *GAPD*: forward GACATCAAGAAGGTGGTGAAGC, reverse GTCCACACCCTGTTGCTGTAG, *SMAD2*: forward AACAGGACGATTAGATGAGC, reverse GACCTGGTTTGTTCAGAGAAGC *SMAD3*: forward TGAGGCTGTCTACCAAGTTGACC, reverse CTAAGACACACTGGAACAGCGG. PCR products were separated on 2.5% agarose gels stained with GelRed (Cambridge Bioscience, Cambridge, UK) and visualised and photographed under UV transillumination.

Real-time quantitative PCR and densitometric analysis

Quantitative real-time RT-PCR was performed using the Lightcycler (Roche Diagnostics Ltd, Welwyn Garden City, UK) as described previously (Hartley et al., 2002). Reverse-transcribed RNA samples were diluted in water, and 1 μl of diluted first-strand cDNA was added to a final volume of 10 μl containing 0.5 $\mu\text{mol/l}$ each of forward and reverse primer in 1 \times Quantitect SYBR Green PCR Master Mix (Qiagen, Crawley, UK). Signal acquisition was performed for each of 45 amplification cycles followed by continuous melt curve analysis to ensure product accuracy. *GAPD*, *SMAD2* and *SMAD3* primers are described above. *FGF2* primers have been described previously (Battersby et al., 2007).

Standard curves for *GAPD*, *SMAD2*, *SMAD3* and *FGF2* were derived using a series of dilutions (1/10 to 1/10,000) of first-strand cDNA from an 18 week ovary. The number of cycles needed to yield a fluorescent signal above background (the cross-over point, Cp) at each dilution was plotted against the log of relative concentration using LightCycler Software (Molecular Dynamics Ltd, Chesham, UK). The dilutions yielded a straight line for each product, confirming that Cp is a good indicator of target concentration across at least 3 orders of magnitude. For quantification, cDNAs were used at 1/10 dilutions with all amplification reactions performed in duplicate. Relative concentrations were calculated from Cp values using the slope of the appropriate standard curve

which was -3.75 for *GAPD*, -3.74 for *SMAD2*, -3.79 for *SMAD3* and -3.19 for *FGF2*. Calculation of *SMAD2*, *SMAD3* and *FGF2* mRNA concentrations were made relative to *GAPD* from the same sample to allow comparisons between cDNAs.

Expression of *KITLG* mRNA were below quantifiable levels using the quantitative PCR method above. For *KITLG* detection conventional 20 μ l PCR reactions containing 1 μ l of cDNA for either *KITLG* or the constitutively expressed control gene *RPL32* were performed and products separated on agarose gels using the concentrations and methods detailed above. Primers used were *hRPL32-F* 5'-CATCTCCTTCTCGGCATCA-3' and *hRPL32-R* 5'-AACCCGTGTGTCAATGCCTC-3', primers to *KITLG* have been described previously (Reber et al., 2006). 35 and 30 cycles of PCR were used to amplify *KITLG* and *RPL32* respectively to avoid saturation. Gels were photographed under UV transillumination and captured gel images imported into ImageJ (NIH, Bethesda) for densitometric analysis. *KITLG* mRNA levels were calculated relative to *RPL32*. Data presented for the expression of *SMAD2*, *SMAD3*, *FGF2* and *KITLG* are mean \pm sem of four independent culture experiments. Significance was determined by *t*-test.

Immunofluorescence of SMADs2 and 3, c-Kit, and activin β A

Paraffin-embedded ovaries were cut into 5 μ m sections and mounted onto electrostatically charged microscope slides (VWR, Poole, UK), dried overnight, and then dewaxed and rehydrated using conventional methods. Antigen retrieval was performed by boiling the slides in citrate (0.01 M) for 2 min followed by immediate cooling in cold water. Endogenous peroxidases were quenched in 3% hydrogen peroxide in methanol for 30 min at room temperature. After a wash in water, slides were transferred into phosphate-buffered saline (PBS) (Gibco) for 5 min and blocked for 30 min in normal goat serum (Diagnostics Scotland, Carlisle, UK) diluted 1:4 in PBS containing 5% BSA. SMAD2 and SMAD3 antibodies (both Zymed, Paisley, UK) diluted 1:60 and 1:100 respectively and c-Kit antibody (without antigen retrieval; Dako Cytomation, Ely, UK) diluted 1:30 were applied to sections at 4 °C overnight in a humidified chamber. SMAD2 and 3 and c-Kit were visualised by tyramide-enhanced fluorescein (Perkin Elmer Life Science, Massachusetts, USA) via a peroxidase conjugated goat anti-rabbit secondary antibody (Dako, Glostrup, Denmark) diluted 1:200. Sections were counterstained with propidium iodide (Sigma) diluted 1:1000. Slides were then washed in PBS and mounted in aqueous mounting medium (Permafluor; High Wycombe, UK). Fluorescent images were captured using a LSM510 confocal microscope (Zeiss). Negative controls were incubated with non-immune rabbit IgG, omitting primary antisera.

To assess the relative nuclear localisation of SMADs 2 and 3 across increasing gestational age, pixel counts were made of unselected images of sections of fetal ovary at 14–16 and 18–19 weeks gestation. To ensure comparability, all sections were immunostained at the same time for each protein, and images were captured using the same laser settings on the confocal microscope. Data were calculated as the number of pixels of nuclear SMAD (i.e., yellow) as a percentage of total SMAD (i.e., green). Correction for the amount of nuclear counterstain (i.e., red, propidium iodide) was also investigated, but this did not alter the findings.

For dual staining of SMAD3 or c-Kit with activin β A, sections were treated as above to the serum blocking stage then blocked with avidin (0.01M; 15 min) and then biotin (0.001M; 15 min; both from Vector Laboratories, Peterborough, UK) with washes in PBS in between. Activin β A antibody (gift from NP Groome) was diluted 1:200 and applied to sections at 4 °C overnight in a humidified chamber. Activin β A was visualised by incubating with the secondary antibody goat anti-mouse Alexa 546 (Molecular Probes, Leiden, The Netherlands), diluted 1:200 in PBS. Slides were blocked for a further 30 min in normal serum and then Smad3 antibody diluted 1:100 or c-Kit antibody diluted 1:30 were applied to sections at 4 °C overnight in a humidified chamber. SMAD3 and c-Kit were visualised as above by tyramide-enhanced fluorescein (Perkin Elmer Life Science). Sections were counterstained with To-Pro (Molecular Probes) diluted 1:1000. Slides were then washed in PBS and mounted before image capture as indicated above.

Immunoblotting

Fetal ovaries or cell pellets (see below) were homogenized in lysis buffer containing 80 mM Tris-HCl (pH 6.8), 1% (vol/vol) glycerol, 1% (wt/vol)

sodium dodecyl sulfate and a Complete Mini protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). Total protein quantification was performed using the BioRad protein assay kit.

Proteins were boiled in a 1:3 volume of 4 \times reduced sample buffer [20% 250 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate, 10% β -mercaptoethanol and 0.1% bromophenol blue]. Ten micrograms of total protein were loaded onto 15% SDS-PAGE gels with high-molecular weight Rainbow protein markers (Amersham Biosciences, Buckinghamshire, UK) run in parallel. Gels were blotted onto polyvinylidene difluoride membrane (Hybond P, Amersham Biosciences) and then blocked in TBS and Tween [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20] containing 5% powdered milk before overnight incubation at 4 °C with primary antibody which was omitted in negative controls. Goat polyclonal antibodies to Smad2/3 and phosphorylated Smad2/3 (Santa Cruz, CA, USA) and β -actin (Santa Cruz) were diluted 1:500, 1:500 and 1:1000 respectively in TBS and Tween 20 containing 5% powdered milk. Bound antibody was detected using horseradish peroxidase-linked secondary antibodies diluted 1:10000 (Santa Cruz). Proteins were revealed and visualized using the enhanced chemifluorescent system (Amersham Biosciences) and

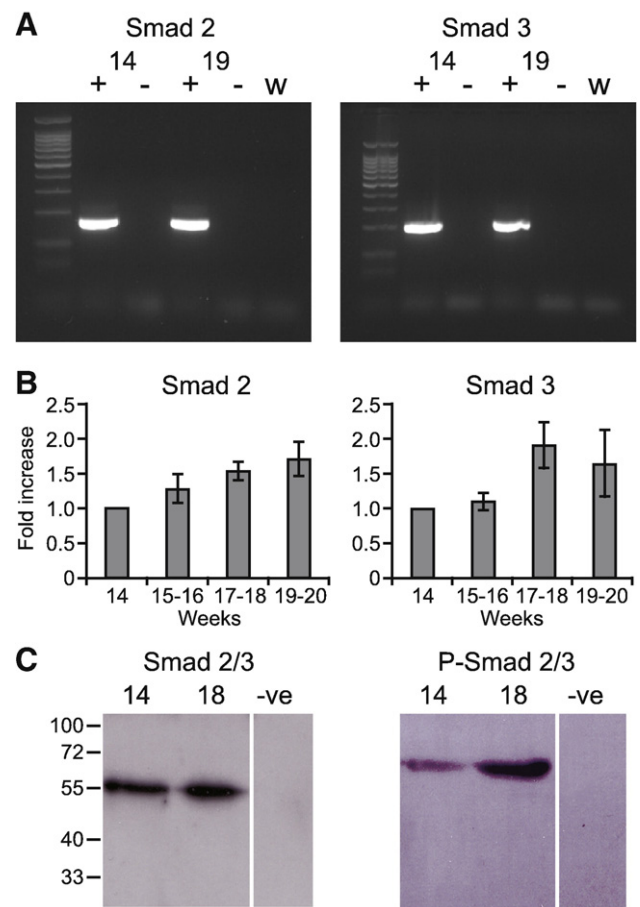


Fig. 1. (A) Expression of mRNA for *SMAD2* and *3* in human fetal ovary. RT-PCR analysis of samples extracted from human fetal ovaries at 14 and 19 weeks gestation. Lanes marked 14- and 19- contained samples in which reverse transcriptase was omitted. W refers to water instead of cDNA in the PCR reaction. (B) Real-time PCR quantification of *SMAD2* and *3* expression in the fetal ovary. Expression of *SMAD2* and *3* mRNA was quantified in human fetal ovary specimens over the gestational range of 14–20 weeks. Data calculated as mRNA expression relative to *GAPD* and expressed as fold increase over expression at 14 weeks. $n=4-7$ per group, mean \pm SEM. There was a significant increase in *SMAD3* but not *SMAD2* mRNA expression over this gestational range ($P=0.02$). (C) Immunoblot for SMAD 2/3 and phosphorylated SMAD2/3 proteins in human fetal ovary at 14 and 18 weeks gestation, and negative control (18 weeks). Molecular weight markers as indicated.

quantified using phosphorimager analysis on the Typhoon 9400 system (Amersham Biosciences) with normalisation for protein loading using β -actin.

Culture of disaggregated human fetal ovary

Fetal ovaries were collected as described above. Both ovaries from a single fetus were placed in Hanks' balanced salt solution (HBSS) (Gibco) and pulled

apart under the dissection microscope using 18 gauge needles. 500 μ l Collagenase Type IV (0.01 g in 1 ml HBSS) (Sigma, Dorset, UK) was added and incubated for 10 min at 37 °C on a roller. Cells were shaken and incubated again for a further 5 min. Fifty μ l DNase 1 (0.007 g in 1 ml HBSS) (Sigma) was added to the suspension and incubated for a further 5 min. The resulting single cell suspension was centrifuged for 5 min at 500 g, the supernatant removed and the cell pellet resuspended in HBSS (Gibco). This wash was repeated twice. The

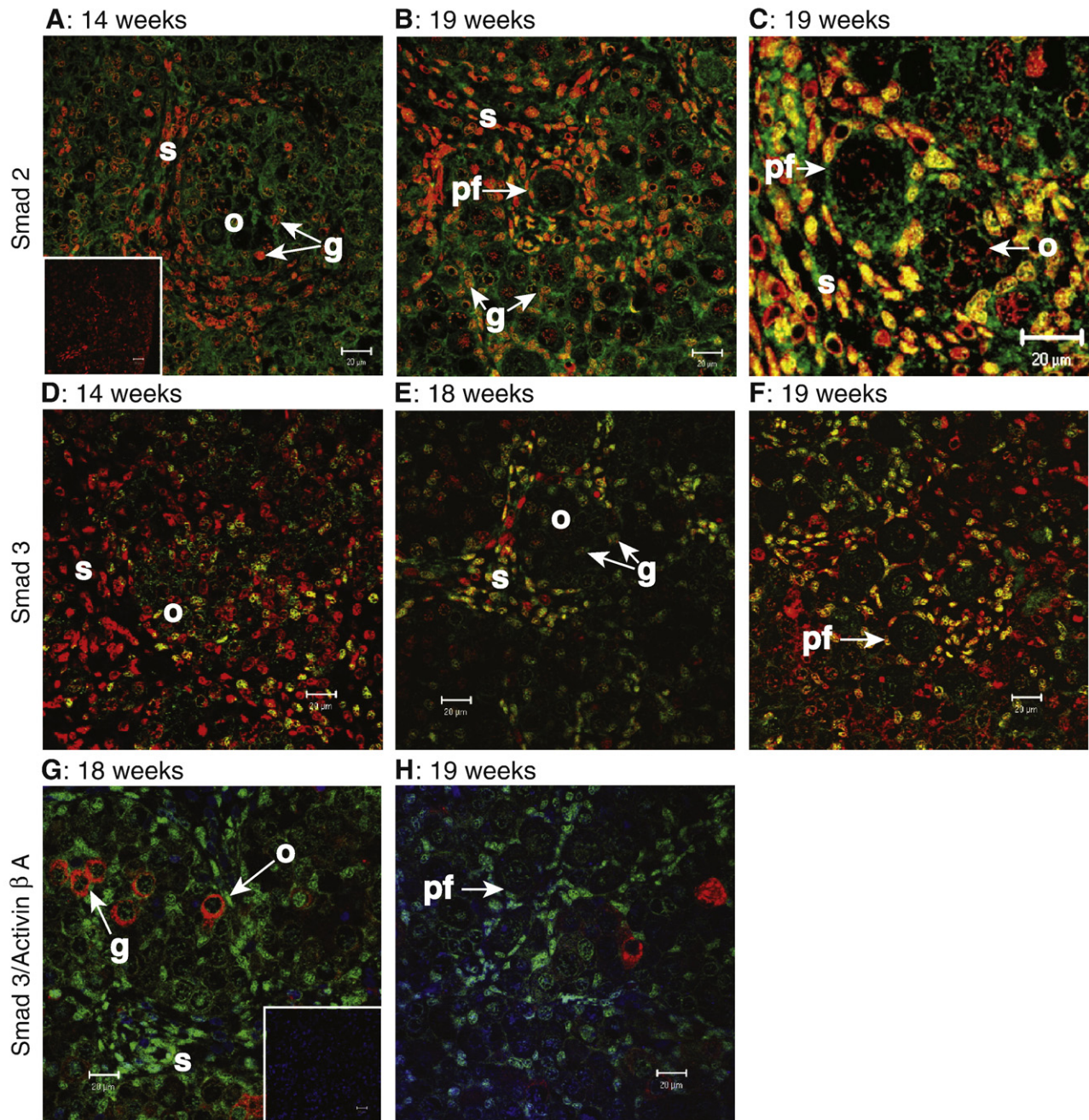


Fig. 2. Immunofluorescent localisation of SMAD2 and 3 and activin β A in the human fetal ovary. SMAD2: (A) 14 weeks gestation, insert shows representative negative control for both SMAD2 and 3; (B and C) 19 weeks gestation. SMAD3: (D) 14 weeks gestation; (E) 18 weeks gestation (F) 19 weeks gestation. Panels B and E show increased stromal cell expression of both SMAD2 and 3 respectively at 18–19 weeks compared to 14 weeks. Panels C and F show SMAD2 and 3 expression in granulosa cells of primordial follicles. Positive staining for SMAD2 and 3 is green and sections were counterstained with propidium iodide (red), thus nuclear SMAD2 and 3 are yellow. (G and H) Dual staining for SMAD3 and activin β A. (G), 18 weeks gestation ovary (insert shows negative control) showing SMAD3 expression in pre-granulosa cells surrounding activin β A-expressing germ cells as well as in somatic cells out-with germ cell clusters. (H) 19 weeks gestation ovary showing that oocytes within primordial follicles do not express activin β A but SMAD3 expression persists from somatic cells within germ cell clusters to granulosa cells of primordial follicles. Positive staining for activin β A is red, SMAD3 green and sections were counterstained with To Pro (blue), thus nuclear SMAD3 is turquoise. s, stroma; g, pre-granulosa cells; o, oocyte; pf, primordial follicle. Scale bars: all 20 μ m.

resultant final fetal ovary cell suspensions were cultured in MEM α medium with phenol red (Gibco) supplemented with 2 mM sodium pyruvate, 2 mM L-glutamine, 1 \times ITS supplement (Sigma) and 3 mg/ml BSA, and with penicillin, streptomycin and amphotericin. Treatments consisted of either 0.4 mg/ml follistatin (Sigma) or 100 ng/ml recombinant human activin A (R&D systems, Abingdon, UK). Cells were cultured for 36 h (for protein extraction) or 24 h (for RNA extraction) in a humidified incubator (37 °C, 5% (v/v) CO₂). At the end of culture, cells were collected, washed in PBS (Gibco) and the pellets snap frozen for protein extraction. For RNA extraction, floating non-adherent cells were collected by centrifugation and resuspended in lysis buffer (RNeasy Kit, Qiagen). The remaining adherent cells were lysed directly in the dish as recommended by the manufacturer and the lysates pooled before RNA extraction as detailed above. Protein analysis by immunoblotting was performed as described above.

Results

SMAD2 and 3 mRNA expression

SMAD2 and 3 expression in the human fetal ovary was demonstrated by RT-PCR across the mid-trimester (Fig. 1A). SMAD2 and 3 mRNA expression was quantified across the gestational range 14–20 weeks by real-time quantitative RT-PCR (Fig. 1B, $n=20$ specimens). This demonstrated a small increase in SMAD2 expression, up to 1.7 ± 0.2 fold at 19–20 weeks compared to 14 weeks, which did not reach statistical significance. SMAD3 mRNA expression also showed an increase which did reach statistical significance ($P=0.02$, Kruskal–Wallis test) with highest expression at 17–18 weeks gestation (1.9 ± 0.3 fold increase vs. 14 weeks). This gestation coincides with the stage at which primordial follicles are first seen.

SMAD2 and 3 proteins in human fetal ovary

Immunoblotting demonstrated the expression of SMAD2/3 and phosphorylated SMAD2/3 proteins in the mid-trimester human fetal ovary (Fig. 1C). Expression of SMADs2 and 3 were localised by confocal immunofluorescence microscopy in all specimens of human fetal ovary examined between 14 and 20 weeks gestation (Fig. 2). Essentially similar results were obtained for both SMAD2 and SMAD3. Three cell types expressing SMADs2 and 3 were identified. Firstly, SMADs2 and 3 was localised to the nuclei of somatic cells in the ovarian stroma between the clusters of germ cells (sex cords). Fewer cells showing nuclear SMADs2 and 3 were identified at 14 weeks gestation compared to later gestation when the great majority of such cells showed nuclear expression of SMADs2 and 3 (Fig. 2, A vs. B and D vs. E for SMAD2 and SMAD3 respectively). Confirming this visual impression, quantification indicated that there was an increasing nuclear localisation of both SMAD2 and SMAD3 with increasing gestation. The proportion of SMAD2 that was nuclear rose from $3.6\pm0.8\%$ at 14–16 weeks to $33.7\pm12.0\%$ at 18–19 weeks, and that of SMAD3 rose from $14.0\pm2.3\%$ to $33.3\pm3.2\%$ (both $p<0.001$, $n=16$ –18 in each case).

Secondly, SMADs2 and 3 were expressed by some somatic cells intermingled with germ cells (Figs. 2B and E). A minority of these cells expressed SMADs2 or 3. Thirdly, SMADs2 and 3

were also localised to the nuclei of pre-granulosa cells surrounding oocytes that had formed (or were in the process of forming) primordial follicles (Figs. 2C and F). No expression was seen in oocytes at any developmental stage.

This pattern of expression was strikingly different to the pattern of expression of activin β A we have previously reported (Martins da Silva et al., 2004). Dual staining for SMAD3 and activin β A was therefore performed. These experiments confirmed previous findings (Martins da Silva et al., 2004), that activin is expressed by the larger germ cells, generally arranged in clusters and located towards the centre of the ovary, but with no expression in oocytes within primordial follicles. This technique allowed the demonstration that the activin-expressing oocytes within the germ cell nests were adjacent to somatic cells expressing SMAD3 (Figs. 2G and H). However the pre-granulosa cells surrounding primordial follicles expressed SMAD3 without expression of activin by the oocyte. The prominent expression of SMAD3 in the somatic cells in the cell streams also appeared not to be adjacent to activin-expressing germ cells.

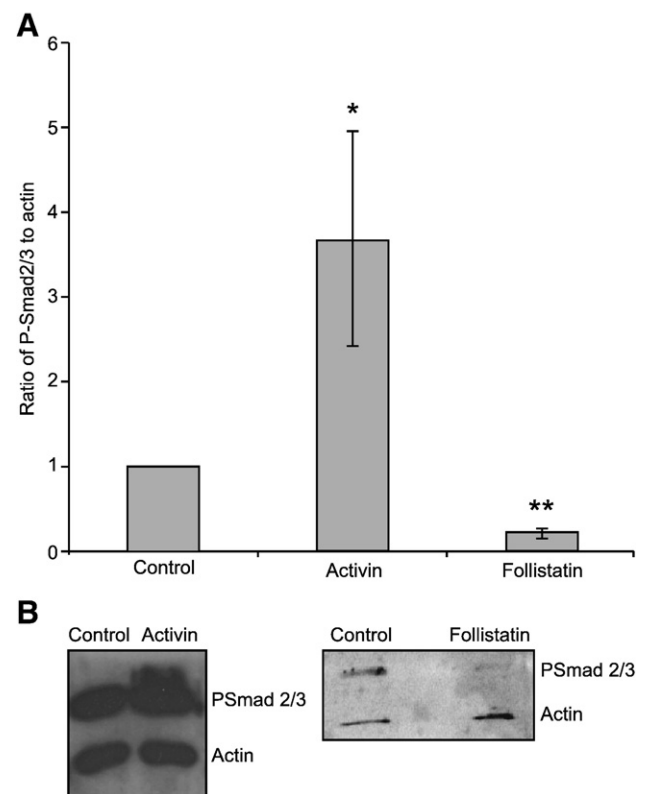


Fig. 3. Immunoblot analysis of the effect of activin β A and follistatin on the expression of phosphorylated SMAD2/3 protein, relative to β -actin. Addition of 100 ng/ml activin increased phosphorylated SMAD2/3 expression to 3.7 ± 1.3 fold greater than control, mean \pm SEM, ($*P=0.02$, $n=5$, Student's t test) whereas addition of 0.4 mg/ml follistatin resulted in a reduction of phosphorylated SMAD2/3 expression to $19\pm6.5\%$ of control, mean \pm SEM. ($**P=0.01$, $n=4$, Student's t test). Panel A shows results of quantification, panels B shows representative immunoblots showing the effect of activin and follistatin respectively.

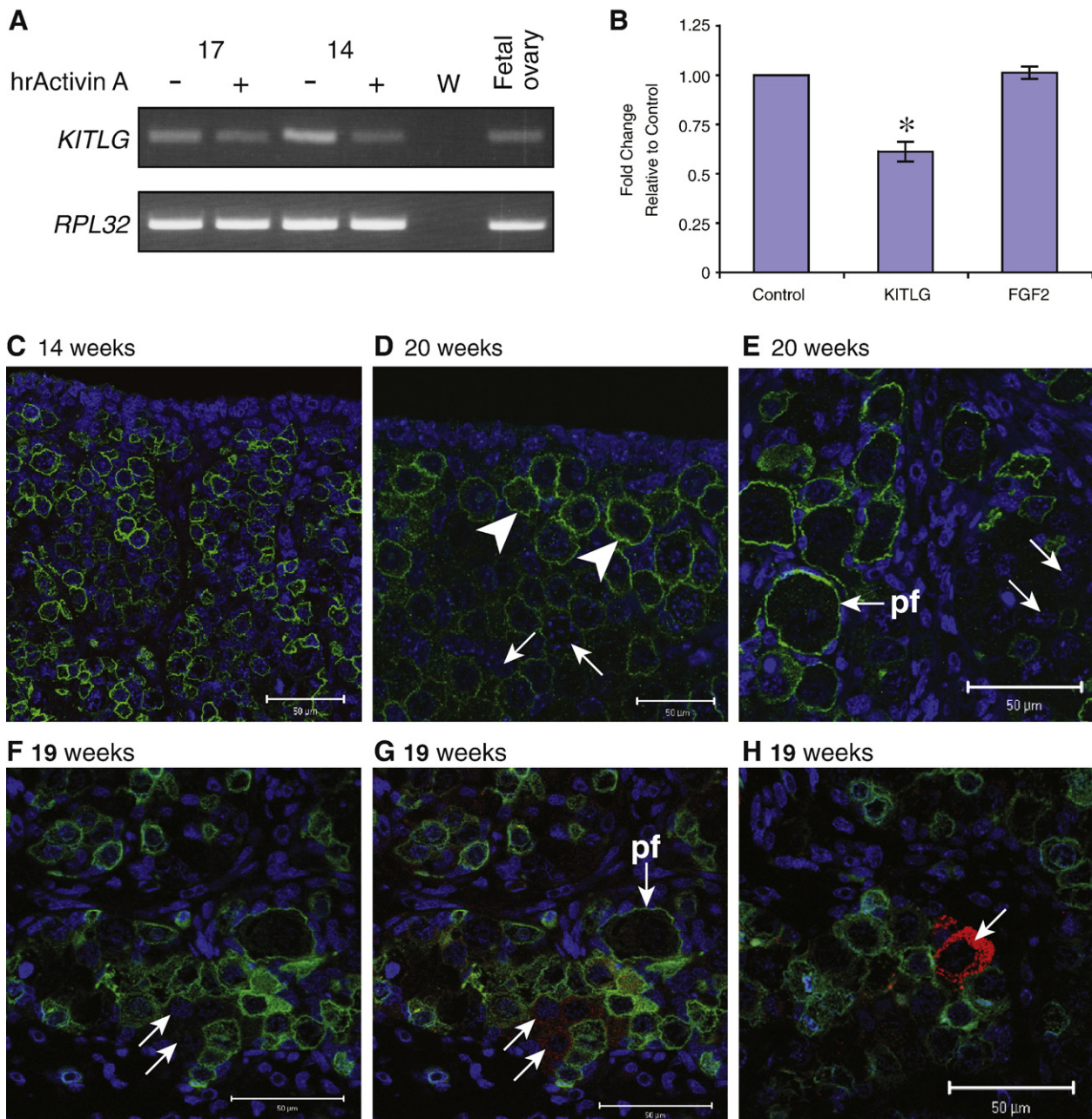


Fig. 4. Repression of *KITLG* expression by activin A signalling and c-Kit/activin β A immunolocalisation. (A) Semi-quantitative PCR analysis showing repression of *KITLG* expression in disaggregated fetal ovary cultures treated with activin A. *RPL32* levels remain constant showing equal amounts of RNA were used in each cDNA synthesis. Two representative experiments are shown, using ovarian tissue of 14 and 17 weeks gestation as indicated. W: water instead of cDNA in the PCR reaction. (B) Densitometric quantification ($n=4$) shows significant decrease in *KITLG* expression in activin A treated ovarian cell cultures compared to control. *FGF2* expression in the same experiments remains unchanged indicating specificity of the effect on *KITLG*. (C–E) Immunofluorescent photomicrographs of human fetal ovaries at 14 and 20 weeks gestation stained for c-Kit (green). Germ cells appear homogeneously immunopositive for c-Kit expression at 14 weeks. At 20 weeks gestation, c-Kit immunopositive germ cells are present near the periphery of the ovary (D, arrowheads) with immunonegative germ cells away from the subepithelial zone (arrows). More centrally at 20 weeks (E) germ cells in clusters do not express c-Kit (arrows), however expression resumes after primordial follicle formation (pf: primordial follicle). (F, G, H) Dual immunofluorescence staining of c-Kit (green) and activin β A (red) in fetal ovary at 19 weeks gestation illustrating that c-Kit is not expressed in the germ cells that show most pronounced activin expression. (F) Separated image of c-Kit expression, (G, H) combined c-Kit and activin β A expression. Arrows, germ cells expressing activin β A but not c-Kit. Sections were counterstained with To Pro (blue). Scale bars: all 50 μ m.

Activin increases and follistatin reduces phosphorylation of SMAD2/3

The effects of activin and follistatin on phosphorylation of SMAD2/3 protein were investigated using an in vitro culture

system. Ovaries were disaggregated and a single cell suspension, containing both germ cells and somatic cells, was treated with activin or follistatin for 36 h. Addition of 100 ng/ml activin increased phosphorylated SMAD2/3 expression to 3.7 ± 1.3 fold greater than control ($P=0.02$, $n=5$) whereas addition of 0.4 mg/

ml follistatin resulted in a reduction of phosphorylated SMAD2/3 expression to $19 \pm 6.5\%$ of control ($P=0.01$, $n=5$, Fig. 3).

Activin represses expression of kit ligand whilst stimulating expression of SMADs2 and 3

Regulation of possible downstream transcriptional targets of activin signalling was also investigated using the in vitro culture system with expression of candidate genes determined by quantitative and semi-quantitative RT-PCR.

Treatment with 100 ng/ml activin A for 24 h resulted in a marked upregulation of *SMAD2* mRNA levels to 6.9 ± 2.7 fold greater than control, ($P=0.05$, $n=4$). A smaller increase in *SMAD3* expression (4.0 ± 1.3 fold greater than control, $n=4$) was also detected, however it did not reach statistical significance.

To determine whether germ cell-derived activin A regulated the transcription of somatic cell produced factors that might in turn influence germ cell growth and/or development we examined the expression of two such known regulators, kit ligand (*KITLG*) and basic Fibroblast Growth Factor (*FGF2*). Expression of *KITLG* mRNA was readily detectable in both fresh and disaggregated ovary but too low to be reliably analysed using the quantitative RT-PCR method used. A semi-quantitative RT-PCR with densitometric analysis was therefore performed. Treatment with activin A reduced *KITLG* transcription to less than two-thirds of that in control ovarian cells ($61 \pm 5\%$ of control, $P=0.04$, $n=4$, Figs. 4A and B). Expression of *FGF2* remained unchanged by activin ($101 \pm 5\%$ of control, $n=4$, Fig. 4B), indicating the specificity of the effect of activin on *KITLG* expression.

Germ cells expressing activin β A do not express c-Kit

As *KITLG* encodes the ligand for the tyrosine kinase receptor c-Kit, we re-analysed the expression of this receptor (Robinson et al., 2001) in the second trimester fetal ovary. At 14 weeks gestation immunofluorescent confocal microscopy demonstrated that all germ cells expressed membrane-bound c-Kit protein (Fig. 4C). At later gestations however, separate populations could be identified based on germ cell size, location and c-Kit expression. Germ cells near the periphery of the ovary continued to express c-Kit as at 14 weeks (Fig. 4D, arrowheads). However expression was more variable away from the subepithelial zone (Fig. 4D) with those germ cells in clusters mostly not expressing c-Kit (Figs. 4D and E). Oocytes that had formed or were in the process of forming primordial follicles re-expressed c-Kit protein at the membrane (Fig. 4E). This pattern of expression, as previously reported (Hoyer et al., 2005), is consistent with downregulation of the c-Kit receptor as germ cells enter meiosis and subsequent upregulation as germ cells form primordial follicles.

As c-kit expression is dependent on kit ligand and activin represses kit ligand expression, we hypothesised that germ cells that express activin would show reduced expression of c-Kit. Germ cells that express c-Kit but not activin include peripheral germ cells at earlier gestations and oocytes in primordial

follicles (Figs. 4C–E) (Martins da Silva et al., 2004) but the situation in germ cells intermediate to these developmental stages is unclear. Dual immunofluorescent staining for activin β A and c-Kit was used to investigate this, and clearly showed that activin β A-expressing germ cells in the germ cell clusters did not express c-Kit, whereas nearby germ cells did.

Discussion

Activin has previously been shown to play a role in the regulation of germ cell proliferation/survival during the developmental period leading up to primordial follicle formation in the human (Martins da Silva et al., 2004) and to promote postnatal primordial follicle assembly in the mouse (Bristol-Gould et al., 2006). The present data indicate this effect is mediated indirectly via somatic cells rather than directly on activin-expressing germ cells, demonstrate that activin signalling is via SMAD2/3 phosphorylation, and indicate that activin downregulates *KITLG* expression, which may constitute the reciprocal somatic to germ cell signal.

Activin binds to ActRIIA and ActRIIB serine–threonine kinase receptors which then recruit a type 1 receptor (ALK4, also known as ActR1A) with subsequent activation of the Smad signalling pathway. The distribution of these receptor subtypes in the human fetal ovary is widespread, with the type II receptors expressed by both germ and somatic cells, although ALK4 was predominantly expressed by the meshwork of ovarian somatic cells surrounding the germ cell nests and by some pre-granulosa cells intermingled with the germ cells (Martins da Silva et al., 2004). Thus, the target cells for activin signalling have, until now, been unclear. Nuclear expression of the ‘receptor Smads’, Smad2 and Smad3, is evidence of active activin or other TGF β factor signalling in that cell. Smad2 and Smad3 have been demonstrated in the postnatal ovaries of mammals (Xu et al., 2002; Drummond et al., 2003; Billiar et al., 2004) and have been demonstrated to play a role in folliculogenesis (Xu et al., 2002). They have both been implicated as important factors in ovarian development and function (Chang et al., 2002; Drummond et al., 2003). However, SMAD2 and SMAD3 have not previously been demonstrated in the human fetal ovary.

In addition to activin, TGF β , GDF9 and Nodal also signal through Smad2 and Smad3 (Shimasaki et al., 2004). One previous study has investigated expression of TGF β 1, TGF β 2 and TGF β 3 and of type I and II TGF β receptors during the development of the human fetal ovary (Schilling and Yeh, 1999). Both ligands and receptors were expressed predominantly by oocytes, i.e. distinctly different from the present data localising SMADs 2 and 3 to somatic cell nuclei, thus it is difficult to reconcile those data on expression of TGF β and its receptors with the present data. Gdf9 is expressed by oocytes in primary follicles in rodents and in primordial follicles in cows and sheep, but is not expressed prior to primordial follicle formation (McGrath et al., 1995; Bodensteiner et al., 1999; Jaatinen et al., 1999; Elvin et al., 2000; McNatty et al., 2001). There is no evidence for the presence of GDF9 in the developing human fetal ovary. Thus GDF9 is also unlikely to

be responsible for SMAD2 and 3 nuclear expression during the stages of development in the present study. There are no data regarding the presence of NODAL in the human ovary.

Mouse models have been generated that are deficient in Smad3 and activin proteins. Two different mouse models with a homozygous deletion in *Smad3* have been developed, with differing effects on fertility. Those with a deletion in *Smad3* exon 8 have reduced fertility compared with the wild-type, with this being due to impaired follicular development (Tomic et al., 2002). Deletion of *Smad3* did not appear to affect the size of the primordial follicle pool in the neonatal period, but altered growth of follicles to antral stages in postnatal life (Tomic et al., 2002). In contrast, mice with a deletion of *Smad3* in exon 2 were fertile (Zhu et al., 1998). It has been proposed that deletion in exon 8 disrupts a region that interacts with the receptors, whereas deletion in exon 2 prevents the production of the active domain of Smad3 (Tomic et al., 2004). Mouse models with mutations in the gene encoding the activin β A subunit are lethal. These mice develop to term but have secondary palate defects and lack whiskers and lower incisors, dying within 24 h of birth due to feeding difficulties (Matzuk et al., 1995). Unfortunately, no conclusions are reached regarding ovarian follicular development. Mouse models lacking the activin β B subunit suffer eye lesions as a result of failed eyelid fusion. These females do not show a disruption in follicular development (Vassalli et al., 1994). Highly increased β A expression was noted in the ovaries of these β B-deficient female mice, suggesting the possibility of functional compensation within the TGF β superfamily. Using a gene knock-in approach, it has been found that activin β B can rescue the craniofacial defects and neonatal lethal phenotype of β A-deficient mice. However, activin β B was unable to substitute for all the functions of β A. The β B knock-in mouse is subfertile and has smaller ovaries than those of controls (Brown et al., 2000). This suggests that activin β A expression is necessary for development of the correct complement of germ cells in the mouse ovary. Adult mice lacking the *Actr11* gene, part of the signalling pathway for activin, have defective reproductive performance and small gonads. Interpretation is complicated by abnormal FSH secretion, in addition to possibly deficient germ cell proliferation (Matzuk et al., 1995).

The results of this study localise both SMADs2 and 3 protein in the human fetal ovary to the nuclei of somatic cells in the ovarian stroma in between the clusters of germ cells. The pattern of distribution for the activin receptors ALK4 and ACTRIIB we have previously reported (Martins da Silva et al., 2004) is very similar to that demonstrated for SMADs2 and 3 in the present study. At 14 weeks gestation, prior to primordial follicle formation, a minority of somatic cells in the ovarian stroma express nuclear SMADs2 and 3. However, by 18 weeks gestation, the majority of these somatic cells show nuclear expression of both SMADs. SMADs2 and 3 were also expressed by some somatic cells intermingled with germ cells, although only by a minority of these cells. These observations confirm the findings observed at the mRNA level, where a near 2 fold increase in *SMAD3* was observed between 14 weeks gestation, i.e. prior to primordial follicle

formation, and 17–18 weeks gestation, during primordial follicle formation. A smaller rise in *SMAD2* mRNA expression did not reach statistical significance. These data are also in keeping with the increase in activin β A expression identified previously in the human fetal ovary (Martins da Silva et al., 2004). Strikingly, SMADs 2 and 3 were also localised to the nuclei of pre-granulosa cells surrounding oocytes that had formed or were forming primordial follicles. Germ cell clusters are surrounded by a basal lamina, separating them from the somatic stromal cells out-with the clusters (Sawyer et al., 2002). This suggests that a stromal somatic cell contribution to granulosa cells is prevented before and during primordial follicle formation, other than in the peripheral sub-epithelial zone. With dual staining, activin β A-expressing oocytes within the germ cell nests were demonstrated to be adjacent to somatic cells expressing SMAD3, suggesting that these oocyte/somatic cell groups may be proceeding to primordial follicle formation. However the pre-granulosa cells surrounding newly-formed primordial follicles expressed SMAD3 without expression of activin β A by the oocyte. It is unclear whether this may reflect persistent SMAD signalling, or whether another TGF β family member is active at that time.

Activin β B expression has also been demonstrated previously in the human fetal ovary. Immunostaining was more widespread than with activin β A, with both germ cells and somatic cells expressing the β B subunit, with the exception of some of those seen at the periphery of the ovary (Martins da Silva et al., 2004). Activin B may therefore be the relevant factor for some of the SMAD2 and 3 identified in the ovarian stroma.

Smad2 and Smad3 expression has been demonstrated in the oocytes and pre-granulosa cells of the mid-trimester baboon fetal ovary, and in the oocytes and granulosa cells of primordial follicles in later gestations (Billiar et al., 2004). This distribution of Smads 2 and 3, predominantly in the somatic cells, is similar to the distribution demonstrated in this current work however we did not detect any expression of SMAD2 or 3 by oocytes in either the single or dual immunostaining experiments.

In addition to demonstrating the distribution of SMADs2 and 3, treatment of fetal ovarian cells with activin led to an increase in phosphorylated SMAD2/3. Follistatin binds activin with high affinity and neutralises its biological effect (Nakamura et al., 1990; Schneyer et al., 1994). Further culture experiments demonstrated that follistatin (in the absence of exogenous activin) reduced phosphorylated SMAD2/3 expression to less than 20% of control. These data therefore confirm that activin signalling involves SMAD2/3 phosphorylation in the human fetal ovary.

In order to identify the downstream transcriptional targets of activin signalling in the fetal ovary we combined *in vitro* culture of disaggregated fetal ovaries with quantitative RT-PCR to examine candidate genes, namely *SMAD2*, *SMAD3*, *KITLG* and *FGF2*. Activin has previously been reported to specifically upregulate *SMAD2* but not *SMAD3* in immortalised human extravillous trophoblast cells (Chen et al., 2003) and in cultured granulosa cells isolated from adult hens (Schmierer et al., 2003). Consistent with these reports, we detected a significant increase

in the levels of *SMAD2* mRNA in our cultures, and a smaller non-significant increase in *SMAD3* expression. While the non-significance of the increase in *SMAD3* may reflect experimental variation, this raises the possibility that treatment with activin selectively upregulates one arm of its signal transduction machinery, and thus biases its effects towards the transcriptional regulation of the subset of genes regulated by *SMAD2* but not *SMAD3*. Treatment of human extravillous trophoblast cells with activin was also found to stimulate the expression of the type I activin receptor (ActRI) (Chen et al., 2003). In conjunction with the upregulation of *SMAD2*, this suggests that activin is capable of promoting a positive feed-forward mechanism in which the cellular response to activin is amplified by increasing expression of its receptors and intracellular signal transduction machinery.

Kitl and *Fgf2* are important regulators of ovarian follicle development, and have been shown to act synergistically to promote primordial follicle development (Nilsson et al., 2001; Nilsson and Skinner, 2004). *Fgf2* has been reported to upregulate the expression of kit ligand, although the reverse does not occur (Nilsson and Skinner, 2004). Treatment with activin did not change the level of expression of *FGF2* mRNA, suggesting that *FGF2* is not a target of activin-mediated transcriptional regulation, at least at this stage of development. In contrast, *KITLG* expression in cultured ovarian cells was reduced by greater than one third in response to treatment with activin. This result is consistent with that recently reported by Pangas et al. who found a significant upregulation of *Kitl* in the ovaries of adult mice in which all activin beta-subunit genes were conditionally deleted in granulosa cells only (Pangas et al., 2007). Treatment of adult mouse granulosa cells with activin repressed *Kitl* expression approximately seven-fold (Pangas et al., 2007). This reduction is somewhat larger than that detected in our cultures, and may reflect that not all bound activin is removed during the disaggregation process, and that germ cells present in our cultures continue to produce activin throughout the course of the experiment. This is supported by the demonstration that treatment with follistatin reduced *SMAD2/3* phosphorylation to 20% of controls, indicating that residual activin is still present or being produced in our cultures and having an effect that can be reduced by follistatin. Indeed, Pangas et al. pre-treated their cultures with follistatin for 24 h and conducted their experiments in the absence of contaminating germ cells (Pangas et al., 2007).

In addition to the demonstration here that somatic cell expression of *KITLG* is repressed by activin, germ cell expression of its cognate receptor c-Kit is also low in the germ cell clusters where activin is expressed. c-Kit is expressed by all germ cells at earlier developmental stages but is reduced at entry to meiosis (Robinson et al., 2001; Hoyer et al., 2005; Hutt et al., 2006; Fig. 4). Expression of c-Kit is again increased as germ cells form primordial follicles. As kit ligand has been shown to upregulate expression of c-Kit (Thomas et al., 2007), it is likely that the repressive effect of activin on kit ligand signalling will promote downregulation of the c-Kit receptor in activin-producing germ cells. This is directly supported by our findings which show that germ cells that express activin β A do

not express c-Kit whereas c-Kit is expressed by other germ cells within the same cluster. Stimulation of the c-Kit receptor in fully grown oocytes has been shown to inhibit progress through meiosis (Ismail et al., 1996, 1997), thus the role of activin in this system may be indirectly to repress c-Kit expression thus allowing germ cells to progress into meiosis until such a time as primordial follicle formation is initiated, and meiotic arrest resumed with the adjacent somatic cells having a key role in this regulatory loop. The involvement of activin in the expression of c-Kit and other known regulators of meiotic progression such as *Sycp1* that contribute to the regulation of primordial follicle formation (Paredes et al., 2005) warrants further investigation.

In conclusion these results provide further evidence that activin signalling via *SMAD2/3* is important in the developing human ovary before and at the time of primordial follicle formation. The mechanism whereby activin enhances germ cell development in the developing human ovary is indirect, with the effects of germ cell-derived activin A mediated by adjacent somatic cells. Somatic cell factors that may mediate the reciprocal somatic cell to germ cell effect include kit ligand, the receptor for which is expressed by germ cells. *SMAD2/3* signalling also appears to be important in the granulosa cells of newly-formed primordial follicles.

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